

Purification and cDNA Cloning of Isochorismate Synthase from Elicited Cell Cultures of *Catharanthus roseus*

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Isochorismate is an important metabolite formed at the end of the shikimate pathway, which is involved in the synthesis of both primary and secondary metabolites. It is synthesized from chorismate in a reaction catalyzed by the enzyme isochorismate synthase (ICS; EC 5.4.99.6). We have purified ICS to homogeneity from elicited *Catharanthus roseus* cell cultures. Two isoforms with an apparent molecular mass of 64 kD were purified and characterized. The K_m values for chorismate were 558 and 319 μM for isoforms I and II, respectively. The isoforms were not inhibited by aromatic amino acids and required Mg^{2+} for enzyme activity. Polymerase chain reaction on a cDNA library from elicited *C. roseus* cells with a degenerated primer based on the sequence of an internal peptide from isoform II resulted in an amplification product that was used to screen the cDNA library. This led to the first isolation, to our knowledge, of a plant ICS cDNA. The cDNA encodes a protein of 64 kD with an N-terminal chloroplast-targeting signal. The deduced amino acid sequence shares homology with bacterial ICS and also with anthranilate synthases from plants. Southern analysis indicates the existence of only one ICS gene in *C. roseus*.

The shikimate pathway is a major pathway in primary and secondary plant metabolism (Herrmann, 1995). It provides chorismate for the synthesis of the aromatic amino acids Phe, Tyr, and Trp, which are used in protein biosynthesis, but also serves as a precursor for a wide variety of aromatic substances (Herrmann, 1995; Weaver and Herrmann, 1997; Fig. 1a). Chorismate is also the starting point of a biosynthetic pathway leading to phyloquinones (vitamin K_1) and anthraquinones (Poulsen and Verpoorte, 1991). The first committed step in this pathway is the conversion of chorismate into isochorismate, which is catalyzed by ICS (Poulsen and Verpoorte, 1991; Fig. 1b). Its substrate, chorismate, plays a pivotal role in the synthesis of shikimate-pathway-derived compounds, and its distribution over the various pathways is expected to be tightly regulated. Elicited cell cultures of *Catharanthus roseus* provide an example of the partitioning of chorismate. Concurrently, these cultures produce both Trp-derived indole al-

kaloids and DHBA (Moreno et al., 1994). In bacteria DHBA is synthesized from isochorismate (Young et al., 1969). Elicitation of *C. roseus* cell cultures with a fungal extract induces not only several enzymes of the indole alkaloid biosynthetic pathway (Pasquali et al., 1992) but also ICS (Moreno et al., 1994). Information concerning the expression and biochemical characteristics of the enzymes that compete for available chorismate (ICS, CM, and AS) may help us to understand the regulation of the distribution of this precursor over the various pathways. Such information is already available for CM (Eberhard et al., 1996) and AS (Poulsen et al., 1993; Bohlmann et al., 1995) but not for ICS.

Isochorismate plays an important role in bacterial and plant metabolism as a precursor of *o*-succinylbenzoic acid, an intermediate in the biosynthesis of menaquinones (vitamin K_2) (Weische and Leistner, 1985) and phyloquinones (vitamin K_1 ; Poulsen and Verpoorte, 1991). In bacteria isochorismate is also a precursor of siderophores such as DHBA (Young et al., 1969), enterobactin (Walsh et al., 1990), amonabactin (Barghouthi et al., 1991), and salicylic acid (Serino et al., 1995). Although evidence from tobacco would indicate that salicylic acid in plants is derived from Phe via benzoic acid (Yalpani et al., 1993; Lee et al., 1995; Coquoz et al., 1998), it cannot be excluded that it is also synthesized from isochorismate. In the secondary metabolism of higher plants, isochorismate is a precursor for the biosynthesis of anthraquinones (Inoue et al., 1984; Sieweke and Leistner, 1992), naphthoquinones (Müller and Leistner, 1978), catalpalactone (Inouye et al., 1975), and certain alkaloids in orchids (Leete and Bodem, 1976).

ICS was first extracted and partially purified from crude extracts of *Aerobacter aerogenes* (Young and Gibson, 1969). Later, ICS activity was detected in protein extracts of cell cultures from plants of the Rubiaceae, Celastraceae, and Apocynaceae families (Ledüç et al., 1991; Poulsen et al., 1991; Poulsen and Verpoorte, 1992). Genes encoding ICS have been cloned from bacteria such as *Escherichia coli* (Ozenberger et al., 1989), *Pseudomonas aeruginosa* (Serino et al., 1995), *Aeromonas hydrophila* (Barghouthi et al., 1991),

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Abbreviations: AS, anthranilate synthase; CM, chorismate mutase; DHBA, 2,3-dihydroxybenzoic acid; ICS, isochorismate synthase.

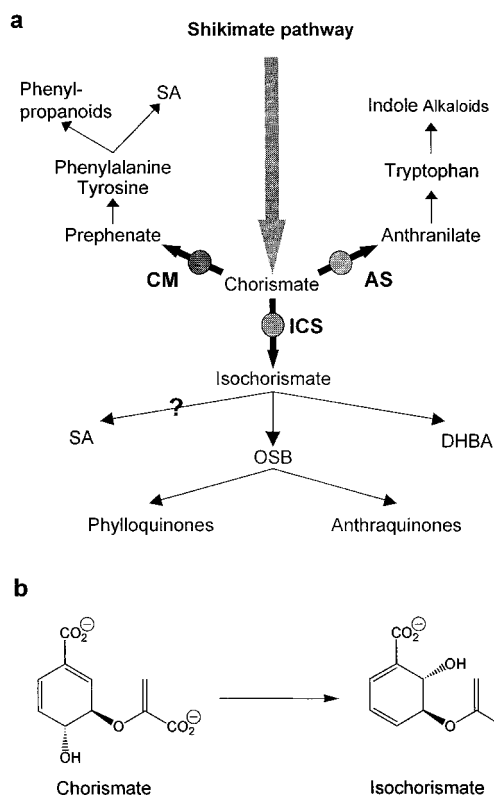


Figure 1. a, Position of ICS in the plant metabolism. SA, Salicylic acid, OSB, *o*-succinylbenzoic acid. b, Reaction catalyzed by ICS.

Flavobacterium K₃₋₁₅ (Schaaf et al., 1993), *Hemophilus influenzae* (Fleischmann et al., 1995), and *Bacillus subtilis* (Rowland and Taber, 1996). Both *E. coli* and *B. subtilis* have two distinct ICS genes; one is involved in siderophore biosynthesis and the other is involved in menaquinone production (Daruwala et al., 1996, 1997; Müller et al., 1996; Rowland and Taber, 1996). The biochemical properties of the two ICS enzymes from *E. coli* are different (Daruwala et al., 1997; Liu et al., 1990). Sequence analysis has revealed that the bacterial ICS enzymes share homology with the chorismate-utilizing enzymes AS and *p*-aminobenzoate synthase, suggesting that they share a common evolutionary origin (Ozenberger et al., 1989).

Much biochemical and molecular data concerning the shikimate pathway in plants have accumulated in recent years (Schmid and Amrhein, 1995; Weaver and Hermann, 1997), but relatively little work has been done on ICS from higher plants. The enzyme has been partially purified from *Galium mollugo* cell cultures (Ledüic et al., 1991, 1997), but purification of the ICS protein to homogeneity has remained elusive, probably because of instability of the enzyme.

Our interests focus on the role of ICS in the regulation of chorismate partitioning over the various pathways. Furthermore, we studied ICS in *C. roseus* to gain insight into the biosynthesis of DHBA in higher plants (Moreno et al., 1994). In this paper we report the first purification, to our knowledge, of ICS to homogeneity from a plant source and the cloning of the corresponding cDNA.

MATERIALS AND METHODS

Chemicals

Chemicals were of an analytical grade from Merck (Darmstadt, Germany). Barium chorismate (60% purity) was purchased from Sigma.

Cell Cultures

Catharanthus roseus (L.) G. Don cell cultures were grown in Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with 30 g/L Suc, as described previously (Moreno et al., 1993). Cell cultures were elicited with *Pythium aphanidermatum* (CBS, Baarn, The Netherlands) filtrate, as described by Moreno et al. (1993).

ICS Assay

ICS (EC 5.4.99.6) activity was determined according to the method of Poulsen et al. (1991) with slight modifications. The incubation mixture (250 μ L) contained 0.1 M Tris-HCl, pH 7.5, 2 mM chorismate, 10 mM MgCl₂, and enzyme extract (125 μ L crude extracts, 10- to 100- μ L column fractions). After the sample was incubated for 60 min at 30°C, the reaction was stopped by the addition of 62.5 μ L of methanol:sec-butanol (1:1, v/v). The samples were centrifuged and analyzed by HPLC. Assay mixtures (250 μ L) for determination of pH optima contained 190 μ L of 0.2 M stock solutions of the various buffers (citrate, pH 4.0–6.0; Bis-Tris, pH 6.0–7.0; Tris-HCl, pH 7.0–9.0; and Gly, pH 9.0–10.0). All other assay components were dissolved in distilled water.

Enzyme Extraction

Cells were harvested by suction 16 h after elicitation, washed once with water, immediately frozen in liquid nitrogen, and stored at -80°C . Six hundred grams of frozen cells was homogenized in a Waring blender equipped with a stainless steel bucket. Extraction buffer (1 mL; 0.1 M Tris-HCl, pH 7.5, 10% glycerol [v/v], 1 mM DTT, 0.2 mM PMSF, 10 mM leupeptin, and 1 mM EDTA) and 50 mg of PVP were added per gram fresh weight. After thawing, the homogenate was centrifuged at 10,000g for 30 min to remove cell debris. The supernatant is referred to as the crude extract.

Purification of ICS

All steps were performed at 4°C. The crude extract was cleared by filtration through a 200- μ m glass-fiber filter. The filtrate was concentrated using a tangential flow ultrafiltration unit (Provario, PAL-Filtron, Breda, The Netherlands) equipped with a 30-kD cut-off membrane. Solid ammonium sulfate (30% saturation) was added to the extract. After the precipitate was stirred for 20 min it was removed by centrifugation at 10,000g for 30 min. More ammonium sulfate was added to the supernatant to 60% saturation. The precipitate was collected by centrifugation

at 10,000g for 30 min. The pellet was dissolved in 50 mL of buffer A (20 mM triethanolamine-HCl, pH 7.5, 10% [v/v] glycerol, 1 mM DTT, and 0.2 mM PMSF), and solid KCl was added to a final concentration of 2 M. After the sample was centrifuged at 13,000g for 15 min, the supernatant was applied to a Phenyl-Sepharose CL-4B column (72 mL, 2.6 × 13.5 cm, Pharmacia) equilibrated in buffer B (buffer A plus 2 M KCl). After the column was washed with 300 mL of buffer B, ICS was eluted with a 700-mL linear gradient from buffer B to A, and then by 150 mL of buffer A, at a flow of 1 mL/min.

Fractions of 10 mL were collected, and those containing ICS activity were pooled and concentrated using the ultrafiltration unit. The concentrate was desalted by gel filtration over Sephadex G-25 columns (PD-10 columns, Pharmacia), equilibrated in buffer A, and applied to a 20-mL Blue A column (Amicon, Beverly, MA). After application the flow was stopped for 30 min to allow binding. The column was washed by reverse flow (0.25 mL/min) with 40 mL of buffer A. ICS was eluted in the same way with a 160-mL gradient from buffer A to 50% buffer B, and 4-mL fractions were collected. Fractions containing ICS activity were pooled, concentrated, and desalted on PD-10 columns equilibrated with buffer C (20 mM triethanolamine-HCl, pH 8.0, 5% [v/v] glycerol, and 1 mM DTT). The desalted sample was applied to a Mono-Q HR 5/5 column (Pharmacia) equilibrated in buffer C. The column was washed with 16 mL of buffer C and ICS was eluted with an 80-mL linear gradient from buffer C to D (buffer C plus 0.5 M KCl). The flow was 0.5 mL/min and fractions of 0.5 mL were collected.

Protein Analysis

Protein concentrations were determined in microtiter plates using the Bradford (1976) microassay method according to the manufacturer (Bio-Rad) with BSA as a standard. SDS-PAGE and native PAGE were carried out on a PhastSystem (Pharmacia) using precast 8% to 25% gradient gels, and proteins were visualized by silver staining (Davis et al., 1986).

Data Evaluation

Kinetic data were fitted to $V = V_{\max} S / (K_m + S)$, where V_{\max} is the maximum velocity, S is the substrate or cofactor concentration, and K_m is the concentration giving a half-maximal rate, using the EZ-FIT curve-fitting computer program (Perella, 1988).

Peptide Sequencing

A band containing approximately 20 μ g of ICS II was isolated from a native PAGE gel. The protein was digested with trypsin and the resulting peptides were separated with reverse-phase HPLC. Sequence analysis was carried with an automated sequenator (model 477A, PE, Applied Biosystems) according to protocols of the manufacturer.

Preparation of PCR Template DNA

A cDNA library (a generous gift from Dr. J. Memelink) was constructed from mRNA isolated from *C. roseus* cell cultures 2 and 4 h after elicitation with yeast extract. The cDNA was prepared with the ZAP-cDNA synthesis kit (Stratagene) and cloned into the λ ZAPII vector. A culture of *Escherichia coli* XL1-Blue, in the exponential growth phase was infected with this cDNA library and grown for 15 min without shaking. An equal volume of double-strength YT medium (Sambrook et al., 1989) was added, and the culture was grown for another 2 h at 37°C with shaking. The culture was incubated for 20 min at 72°C and centrifuged for 10 min at 14,000g. Phage DNA was precipitated from the supernatant with ethanol, redissolved in 200 μ L of water, and used for PCR without further processing.

PCR

For PCR a degenerated sense primer (LPT42) against peptide 3 (Fig. 4) was designed with the sequence 5'-GCIGGICCTIGGITT(C/T)TT(C/T)GG(A/C/T/G)GG-3'. As an antisense primer, the T7 primer complementary to a λ ZAPII vector sequence close to the poly(A⁺) tail of the cDNA was used.

PCR reactions were performed with 5 μ L of template DNA, 100 μ M deoxyribonucleotide triphosphates, 2 mM MgCl₂, 20 pmol of the T7 primer, 200 pmol of the LPT42 primer, and 1 unit of Goldstar *Taq* polymerase (Eurogentec, Seraing, Belgium) in the buffer supplied by the manufacturer. PCR amplification was performed with 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 61°C, and 3 min of elongation at 72°C, except for the last cycle when elongation was extended to 10 min. A 520-bp PCR product was isolated from a 1% agarose gel and cloned into the pCR2.1 vector (Invitrogen, San Diego, CA), resulting in pCRIS3.

cDNA Library Screening

For the *C. roseus* cDNA library, 450,000 plaque-forming units were screened with a 440-bp *EcoRI/PvuII* fragment of pCRIS3 labeled with [γ -³²P]ATP. Prehybridization and hybridization were performed at 58°C in 6× SSC containing 5× Denhardt's solution (Sambrook et al., 1989), 0.5% SDS, and 100 μ g/mL herring-sperm DNA. Filters were washed in 0.5% SSC with 0.1% SDS at 58°C. Positive plaques were isolated and subjected to a second screening under the same conditions. Positive plaques from this second screening were excised in vivo according to the protocols of the manufacturer (Stratagene).

Sequence Analysis

cDNA clones were sequenced with 17-mer oligonucleotides using the Prism sequencing kit (Perkin-Elmer). The reaction products were resolved on an ABI PRISM 310 genetic analyzer (Perkin-Elmer). Sequences were analyzed

using software from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

Southern Hybridization of Plant DNA

DNA (20 μ g) was extracted from leaves and digested with *EcoRV* or *Bam*HI. The digest was electrophoresed on a 0.8% agarose gel. Southern blotting and hybridization were performed on a Hybond-N⁺ membrane as described by the manufacturer (Amersham). The full-length ICS cDNA was used as a probe.

RESULTS

Purification of ICS

Cultures of *C. roseus* were elicited 5 d after inoculation, when the cells were in the exponential growth phase. Sixteen hours later, the cell mass was harvested (Moreno et al., 1994). The fungal elicitor induced ICS (EC 5.4.99.6) to a high level. ICS was purified from these cultures as summarized in Table I. Ammonium sulfate precipitation yielded good and reproducible fractionation without loss of ICS activity. Hydrophobic interaction chromatography on Phenyl-Sepharose produced good separation of ICS activity from more than 90% of the protein. Dye-affinity chromatography on a Blue A column proved to be a crucial purification step. This chromatographic step resulted in a 15-fold increase in specific activity.

Anion-exchange chromatography on a fast-protein liquid chromatography Mono-Q column was used as the next purification step. On this column ICS activity was separated into two peaks (ICS I and ICS II), as shown in Figure 2. The specific activities were increased 532- and 754-fold for ICS I and II, respectively, relative to the combined activities in the crude extract. ICS I and II had an activity ratio of 1:2, a number found in several independent purifications. Reinjection of either ICS resulted in the occurrence of only the injected ICS in the chromatogram, indicating that one isoform is not a breakdown product of the other.

After a final purification by native PAGE, ICS II was obtained in a pure form (Fig. 3b). Measurement of ICS activity in slices from neighboring nonstained lanes showed that only the band migrating at approximately 96 kD contained ICS activity. SDS-PAGE of ICS II revealed that this protein is about 64 kD (Fig. 3d). The purest Mono-Q fraction containing ICS I showed several bands on

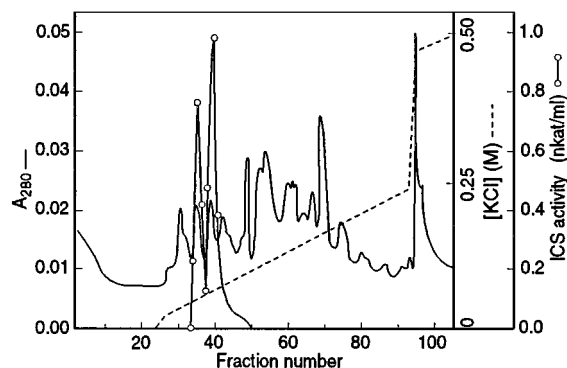


Figure 2. Elution profile of the Mono-Q column used in the purification of ICS from elicited cell cultures of *C. roseus*.

SDS-PAGE (Fig. 3c), as expected from the native PAGE separation (Fig. 3a). One of these bands is a protein of approximately 64 kD (Fig. 3a, arrow), the intensity of which correlated with the ICS activity in the various Mono-Q fractions tested.

Biochemical Characterization

Both isoforms showed an identical pH dependency with a broad pH optimum between 7.0 and 9.0 and 50% of the maximal activity at pH 6.5 and 10. The presence of Mg^{2+} was essential for product formation. Separate incubations with other divalent ions (Mn^{2+} , Co^{2+} , Ni^{2+} , Ca^{2+} , Zn^{2+} , Ba^{2+} , Cu^{2+} , Cd^{2+} , and Fe^{2+}) in a concentration of 10 mM did not result in enzyme activity of either isoform. ICS activity of both isoforms was not inhibited by the presence of Tyr, Phe, or Trp in the assay mixture.

Kinetic studies of the ICS isoforms were carried out separately for each isoform with Mono-Q fractions completely free of AS and CM activities. The dependence of the activity on the chorismate concentration was determined at 10 mM $MgCl_2$. Both isoforms showed standard Michaelis-Menten kinetics for chorismate. The K_m values for chorismate were 558 ± 5 and 319 ± 41 μ M for ICS I and II, respectively. Typical saturation curves were obtained for the enzyme activity of both isoforms as a function of Mg^{2+} concentration (2 mM chorismate). The saturation curves followed Michaelis-Menten kinetics, with K_m values of 1.27 ± 0.36 and 1.63 ± 0.12 mM for ICS I and II, respectively.

Table I. Purification of ICS from 600 g of elicited cells of *C. roseus*

Fraction	Activity	Protein	Specific Activity	Yield	Purification Factor
	pkat	mg	pkat/mg	%	
Crude extract	26,903	847	32	100	1
(NH_4) ₂ SO ₄ pellet	28,688	390	74	106	2.3
Pool Phenyl-Sepharose	11,581	35.4	327	43	10.3
Pool Blue A	6,382	1.28	4,986	23.7	157
Mono-Q 36 (ICS I)	759	0.045	16,867	2.8	532
Mono-Q 40 (ICS II)	980	0.041	23,902	3.6	754

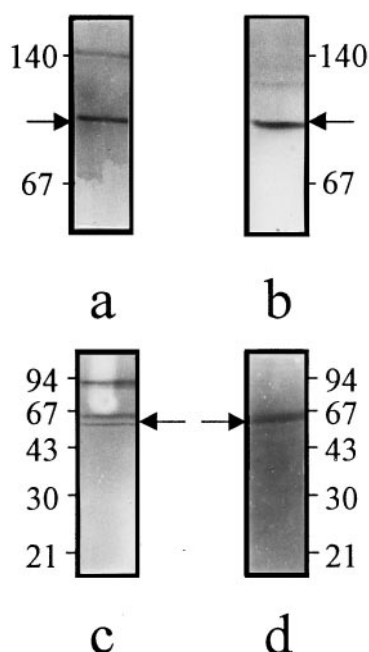


Figure 3. Purified ICS isoforms from elicited cell cultures of *C. roseus*. Proteins were analyzed on a PhastSystem using precast 8% to 25% gradient gels. Proteins were visualized by silver staining (Davis et al., 1986). ICS I (left) and ICS II (right) were analyzed on native PAGE (a and b) and SDS-PAGE (c and d) gels. The positions of the molecular mass markers used are indicated in kDa. The arrows indicate the position of the ICS protein bands.

cDNA Cloning of ICS

The protein band containing ICS II was isolated from a native PAGE gel and digested with trypsin, which yielded about 50 peptides. Five peptides were isolated and sequenced. One of these peptides (no. 3) displayed high homology to bacterial ICS sequences (Fig. 4). Therefore, a degenerated primer was designed against this peptide. PCR on a cDNA library of elicited cell cultures of *C. roseus* with this primer and the T7 primer, complementary to a λ ZAPII sequence close to the poly (A⁺) tail of the cDNA, yielded a fragment of 520 bp. This fragment was cloned and sequenced and proved to contain the complete coding sequence for peptide 3, confirming that we had amplified part of an ICS cDNA. A 440-bp fragment of the amplified DNA was used to screen the cDNA library. Screening of 450,000 plaques identified 52 independent, positive plaques.

Twelve randomly chosen plaques were isolated and subjected to a second screening with the same 440-bp probe. This resulted in the identification of seven independent, positive plaques. These were excised in vivo and partially sequenced (400 bp). All clones contained the previously amplified sequence at the 3' end. The longest clone had an insert of approximately 2.1 kb and contained an ATG codon near the 5' terminal end. The region around this ATG (TCCAATGGC) closely resembled the consensus sequence for translation initiation in plants (Lütke et al., 1987). The ATG codon was preceded by a sequence (TTCTCTCTCTCTCTCTCTCTCCCATCCA) with an 11-

fold repetition of TC, which is rather unusual for a coding sequence. These features all point to the conclusion that translation starts at this point in the sequence. The cDNA with a complete length of 2078 bp (accession no. AJ006065)

C. r. ICS	MAITGHCVAFHTDLSTRKSFFSNMNNSSLFRRKSTNIVTRKKYIFCSTSLSM	55
A. t. ICS1	MTAVLS-----	6
E. c. MenF	MQSLTT-----	6
R. g. ASa2	MITLVNE-TPPLT--RSQLPSTFRVSSAASVNFNDRVATSRWR-----PNSLSL	46
C. r. ICS	NGCNGDPAPVGTITETRLPAVSTPALAMERLSSAVANLKSTLPSAQSGIIRL-E	109
A. t. ICS1	-----BAAATERLISAVSELKSPSPSSGIVRL-Q	36
E. c. MenF	-----ALENLRHLRSLQEIATPGIR--VIDI-P	31
R. g. ASa2	TTSSYRLRLTKCAASASTASTASPSPPSLVDQSANFHEA--SKGNLIPLYR	99
C. r. ICS	VPIEKHEALDWLSQDQKNLLPRCYFSGRSQVTSDFSTNDLTNRNGSAANGHL	164
A. t. ICS1	VPIDQIGALDWLQANE--IQRCFFSRSDVGRPDLL--DLANENGNGNGN--	86
E. c. MenF	FPKDAFDALSWLASQ-----TYPQFYWQ-----RNGDEAVVL	67
R. g. ASa2	CIFSDHLTPV-----LAYRC-LVKEDDRDAPSLFESV--EPGSQASSI	140
C. r. ICS	QRISTSSDDKNLVSAGVGSVAVL-----RSPNPFSDDWLSIKRFLSK-NCPL-	212
A. t. ICS1	---GTVSSDKNLVSAGVGSVAVF-----RDLDPFSDHWRIRRLSS-TPPL-	131
E. c. MenF	-----GATITF-----TSLDQ-----AQRLRQ-HPHHA	90
R. g. ASa2	GRYSVVGQAFAIEIVAKENMTILDHEGGQRTEQFVEDPMDVPRRIMEGWKPLI	195
C. r. ICS	-IR-----AYGAIRFDARPHIAPENKARFSYFVVPQVE-----FD	247
A. t. ICS1	-IR-----AYGMRFDPNGLIAVEWEPGAFYFVQVE-----FN	166
E. c. MenF	DLR-----IWGLNAFDESQ-----GNL--LLPRLE-----WR	115
R. g. ASa2	DELPEAFCGWVGYSYDITVRYVEKKLPPFSAPTDDNLPDVLGLYDDVIVFD	250
C. r. ICS	ELHGSSMIAATVANDN--ALSILTYQQAIVALQTTMEQVSSVTS-----KIRQDV	294
A. t. ICS1	EFGSSSMIAATTANDD--ELSWTLENALIALQETMLQVSSVVM-----KLRNRS	213
E. c. MenF	RCGGKATRLRLTFSSES--SLQ--HDAI-----QAKFIAITLVS-----IKPLFG	155
R. g. ASa2	HVEKKAFFVHWRLDQYSSVAEAYNDGMNRLENLVSRLVDFVFKLRSGSLKHT	305
C. r. ICS	SHTSLVSKANIPDRTSWDLTNRVLEIEGNYSPILTKVVLARRSQVITTSIDDEL	349
A. t. ICS1	LGVSILSKNHVPTKGAYFPAVEKALEMINQKSSPLNKKVILARNRSPITITDIDPI	268
E. c. MenF	LHLTTTREQHWPKTKWTQLILEATKTIAE--GELDKVILARATDLHFASPVNAA	208
R. g. ASa2	RHEGPKLERSMTSEAYKFAVLEAKEHILA--GDIFQIVLSQRFERFRTA--DPP	356
C. r. ICS	AWLSFKADGKDAYQFCLOPHEAPAFIGNTPQELFGDQQLTVFSEALAAATARGE	404
A. t. ICS1	AWLAQLQREGHDATQFCLOPFGAPAFIGNTPERLFRTRLGVCSEALAAATPRAA	323
E. c. MenF	AMMAASRLNRLNCTHFYMAFDGNAFLGSSPERLWRERDKALRTEALACTVANNP	263
R. g. ASa2	EIYRSLRVNPSPYMTYLAQARGC--ILVASSFEILTIVKRRKINTNRILATIRKG	410
C. r. ICS	SDSLDLQMAHDLFSSPKDNHFAIVRENIRKQLDAICTS--VETPEMKSVRLKR	457
A. t. ICS1	SSARDMEIERDLTSPKDDLFKSVRENIRKKNXICDR--VUVKPKVIRKLAR	376
E. c. MenF	DDKQAGQLGEWLMADDKNGRNNHVVVEDICQRLQADTQT--LDLVPQVLR-LRK	315
R. g. ASa2	TRKEDLVFEKELLNDEKQCAVHLVLDGRNDVGVKSVKVEKLMHIEHYS	465
C. r. ICS	IQLHYARFAGRLRSEDEEKILASLHPITPAVCGFPMEDARKFYAENEMEDAGLYA	512
A. t. ICS1	VQHLYSQLAGRLTKEDDEIKILAAHLPTAVCGLPAAEARLLIKIESFDRGYA	431
E. c. MenF	VQLRRRCITWSLKNKADDVIL-CLHQQPTAAVAGLPDLARQFIARHPPTREWYA	369
R. g. ASa2	VMISSSTVTGELLDLHTLSWDALRAALPVGTVSGAPKVKAMEIDKLEVTFRGYPG	520
C. r. ICS	GFVGFPG-CAQSDFSVGSIALICKDAG-ALIV-----KGLGVV	549
A. t. ICS1	GFVGFPG-GESEFAVGIRSAIVKGLG-ALIV-----KGLGVV	468
E. c. MenF	GSAGYLS-LQQSEFCVSLRSKISGVNV-RL-Y-----KAGIV	405
R. g. ASa2	GGFGISFTGDLIDIALALNTWVFQATRYDTHYSYKVDKREWIAHLOAGAGIV	575
C. r. ICS	BGSDPALEWQELKASQFQKIMKLEAP-----ALK	580
A. t. ICS1	AGSDPSSSEWNELDLKIQQTKSIEYEATTSLOAIN	503
E. c. MenF	RGSDPEQWQEIQNKAAAGRLTLQME	431
R. g. ASa2	ADSDPADEQRECEKAAALARAIDLAESSPIE--K	608

Figure 4. Alignment of the ICS from *C. roseus* with the menaquinone-specific ICS from *E. coli* and the α -subunit of an AS from *Ruta graveolens*. The putative ICS from *Arabidopsis* recently deposited in the EMBL databank by H. Meng, G. Peter, and G. Pullman is included in the alignment for a comparison. Sequences shown are *C. roseus* ICS (C. r. ICS; accession no. AJ006065), *Arabidopsis* putative ICS (A. t. ICS1; accession no. AF078080), *E. coli* MenF (E. c. MenF; accession no. D90857), and *R. graveolens* ASa2 (R. g. ASa2; accession no. L34343; Bohlmann et al., 1995). Residues identical to those in *C. roseus* ICS are in bold, perfectly conserved residues are indicated with asterisks, and well-conserved residues are indicated with periods. Elements involved in Trp inhibition of AS (Caliguri and Bauerle, 1991; Bohlmann et al., 1995) are indicated with "<". Residues essential for AS activity (Caliguri and Bauerle, 1991; Bohlmann et al., 1995) are indicated with ">". Peptides isolated and sequenced from the purified *C. roseus* ICS are indicated with <>.

contained an open reading frame of 1740 nucleotides encoding a protein of 580 amino acids. The calculated molecular mass was 64 kD and the pI was 7.7. All five peptide sequences obtained from the purified protein were present in the deduced amino acid sequence and are indicated in Fig. 4. The 3'-untranslated region was of varying lengths in different clones, which indicates that different polyadenylation sites were used in the process of poly(A⁺) tail attachment.

The predicted protein is approximately 30% identical (40% homologous) with ICS from bacteria, with the most homology in the C-terminal region (Fig. 4). There is also 30% homology (15% identity) between the ICS protein and the α -subunits of AS genes cloned from *Arabidopsis* (Nigoyi and Fink, 1992) and *R. graveolens* (Bohlmann et al., 1995; Fig. 4). Also, the homology is strongest in the C-terminal part of the protein.

ICS is probably a plastidic enzyme, since the N-terminal region has the characteristics of a chloroplast transit peptide, i.e. a high number of basic amino acids, Ser residues, and hydroxylated amino acids (Von Heijne et al., 1989). There is no obvious cleavage site (Gavel and Von Heijne, 1990).

Figure 5 shows a Southern blot of *C. roseus* leaf DNA probed with the complete ICS cDNA. The DNA was digested with enzymes that did not cut in the cDNA. A single prominent, hybridizing band was detected in all of the lanes. This indicates that there is only one ICS gene in *C. roseus*.

DISCUSSION

Using a six-step purification method, we purified to homogeneity, for the first time to our knowledge, an ICS from a plant source. Sequence information from the protein led to isolation of a cDNA for ICS.

The biochemical characteristics such as substrate affinity, pH optimum, and cofactor requirements of ICS from *C.*

roseus are similar to those in *Galium mollugo* (Ledüç et al., 1997) and *Rubia tinctorum* (L.J.P. van Tegelen, unpublished results). The ICS in *C. roseus* exists in two isoforms. The identical ratios between the isoforms in several independent purifications and their stability upon reinjection indicate that the dual state is not an artifact from the purification procedure. In elicited *R. tinctorum* cell cultures, ICS is also present in two isoforms, although in a different ratio (L.J.P. van Tegelen, unpublished results). In *G. mollugo* only one ICS activity was found (Ledüç et al., 1997). The bacteria *B. subtilis* and *E. coli* have two ICS proteins encoded by separate genes (Daruwala et al., 1996; Müller et al., 1996; Rowland and Taber, 1996). The ICS proteins have different functions, one in the biosynthesis of siderophores and the other in the biosynthesis of menaquinones (Müller et al., 1996), and they have quite different biochemical characteristics (Daruwala et al., 1997). In contrast, the isoforms in *C. roseus* do not differ very much in their biochemical properties.

Some enzymes in the shikimate pathway also exist in isoforms that are differentially regulated, as only one isoform is inducible by wounding or elicitation (Keith et al., 1991; Muday and Herrmann, 1992; Görlach et al., 1995). The ICS isoforms in *C. roseus* are both induced after elicitation.

Sequencing of the cDNA revealed that all peptides of the ICS protein sequenced are part of the deduced amino acid sequence. This proves that the cDNA really encodes ICS, a conclusion substantiated by the homology to the bacterial ICS proteins. The cDNA encodes a protein of 64 kD, which is equal to the molecular mass of the purified protein. The similarity was rather unexpected because the transit peptide is normally cleaved off upon transport into the plastid. Because no obvious cleavage site can be predicted from the sequence (Gavel and Von Heijne, 1990), it is impossible to estimate the molecular mass of the mature protein from the cDNA.

We did not use very stringent hybridization conditions in the screenings to avoid missing heterologous cDNAs possibly encoding ICS I. Nevertheless, the coding regions in all of the cDNAs analyzed were identical. This suggests that the two isoforms are encoded by the same cDNA and that the differences are caused by posttranscriptional modifications. This idea is substantiated by Southern analysis, which indicates that there is only one ICS gene.

The encoded protein possesses a plastidic import signal and is thus probably located in plastids. Almost all enzymes of the shikimate pathway contain a plastidic import sequence and are present in the plastids (Weaver and Herrmann, 1997). Location of ICS in the plastids makes sense because its substrate, chorismate, is biosynthesized there. Operation of ICS in the cytosol would require a translocator transporting chorismate out of the plastids. The presence of a cytosolic chorismate mutase (Eberhard et al., 1996) is the only indirect evidence for the existence of such a translocator.

The homology of the ICS protein with AS α -subunits (Fig. 4) points to a common reaction mechanism. The reaction catalyzed by ICS is an unusual 1,5-substitution. In bacteria, studies with ¹⁸O-labeling have shown that the

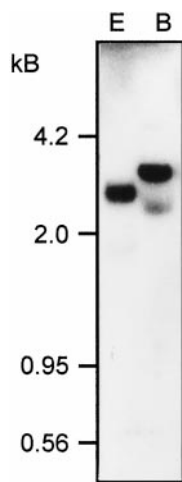


Figure 5. Copy number of the ICS gene in *C. roseus*. DNA from leaves was digested with *EcoRV* (E) or *BamHI* (B), separated, and blotted onto a Hybond-N⁺ membrane. Hybridization was done with the full-length ICS cDNA.

incoming hydroxyl group is derived from the solvent and not from intramolecular transfer (Gould and Eisenberg, 1991). A Mg^{2+} -coordinated transition state has been suggested for the conversion of chorismate to isochorismate. The α -subunits of AS catalyze a reaction very similar to that of ICS, except that the incoming nucleophile is ammonia. NMR and electron paramagnetic resonance studies of AS indicated that Mg^{2+} interacts with chorismate at the active site of the enzyme (Koslowski et al., 1995). The fact that only Mg^{2+} was able to activate the enzyme suggests that the ICS from *C. roseus* has specific affinity for Mg^{2+} in its active site.

All amino acids previously identified as being involved in the allosteric feedback inhibition by Trp in AS (Caligiuri and Bauerle, 1991; Bohlmann et al., 1995) are lacking from the ICS protein and, indeed, ICS is not inhibited by Trp or by any other aromatic amino acid. However, all residues considered essential for activity of AS (Bohlmann et al., 1995) are identical in ICS, with the exception of residue 487, which is an Ala conserved in all ICS proteins cloned so far.

ICS activity in the *C. roseus* cells is measurable only after elicitation (Moreno et al., 1994). Excessive drainage of chorismate into isochorismate-derived products under these conditions may be prevented by the low affinity of ICS for chorismate (558 and 319 μM for ICS I and II, respectively), as compared with that of AS (67 μM ; Poulsen et al., 1993). No data are available for CM from *C. roseus*. The fact that ICS activity in *C. roseus* cells is measurable only after elicitation suggests that the flow of chorismate in the direction of isochorismate is controlled by differential gene expression. Future experiments will therefore focus on the regulation of expression of the chorismate-utilizing enzyme and its impact on metabolic trafficking at this important crossroad in plant metabolism.

NOTE ADDED IN PROOF

After submission of this paper, a putative ICS cDNA clone from *Arabidopsis* was submitted to the EMBL database (accession no. AF078080) by H. Meng, G. Peter, and G. Pullman

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